EXTRACTION OF PAPAIN ENZYME FROM PAPAYA LEAVES USING ENZYME ASSISTED METHOD

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ABSTRACT

Papain is highly appreciated in medical field that prevents several chronic diseases such as cardiovascular disease, cancer and diabetes. Therefore, the objective of the research is to extract papain enzyme from papaya leaves using enzyme assisted method and determine papain activity. The Cellullase assisted extraction process was optimized by varying different parameters such as pH of extraction process, Cellulase concentration, Solid to Liquid ratio, incubation time and incubation temperature. Each 5 g of papaya leaves were ground and mixed with acetate buffer at a different Solid to Liquid ratio (1:5 - 1:25 g/ml) and adjusted with different pH (3-8). Cellulase was quantified and dispersed in acetate buffer (Na_2HPO_4—citric acid) in different concentration (0.5 - 2.5 mg/ml).The enzymatic pretreatment was carried out and continued for enzyme assisted extraction process for various temperature and time (50-70^oC and 1-5 h respectively). The optimal extraction conditions that satisfied the above constraints were found to be at pH 7, 1.5 mg/ml of Cellulase, solid to liquid of 1:10 (g/ml), an extraction time of 4 h and at an extraction temperature of 65^oC. Under the optimum conditions, the extraction yield of papain was successfully achieved as much as 3.8018 µmole with 2.0910 Units/ml in Cellulase treated sample. Therefore, the potential for the papain enzyme in medical field can be fulfilled using enzyme assisted extraction.

ABSTRAK

Papain sangat dihargai dalam bidang perubatan yang mencegah beberapa penyakit kronik seperti penyakit jantung, penyakit kanser dan diabetes. Oleh kerana itu, tujuan dari penelitian ini adalah untuk mengekstrak enzim papain dari daun betik menggunakan kaedah pendekatan enzim dan menentukan aktiviti enzim papain. Proses pengekstrakan melalui kaedah pendekatan Cellullase telah dioptimumkan dengan mempelbagaikan parameter yang berbeza seperti pH untuk proses pengekstrakan, kepekatan selulase, nisbah pepejal kepada cecair, masa inkubasi dan suhu inkubasi. Setiap 5 g daun betik dikisar dan dicampur dengan buffer asetik pada pelbagai nisbah pepejal kepada cecair(1:5 - 1:25 g/ml) dan disesuaikan dengan pH yang berbeza (3-8). Selulase telah disukat dan dimasukkan dalam buffer asetat (Na_2HPO_4-sitrat asid) dengan kepekatan berbeza (0.5-2.5 mg /ml). Rawatan awal enzimatik dilakukan dan dilanjutkan untuk proses pengekstrakan melalui penambahan enzim dengan pelbagai suhu dan masa (50-70^oC dan 1-5 jam untuk masing-masing). Selanjutnya proses diteruskan dengan penapisan dan sentrifugasi. Parameter optimum diperolehi seperti berikut: pH untuk proses pengekstrakan adalah 7, 1.5 mg / ml untuk Selulase, 1:10 untuk nisbah pepejal kepada cecair, inkubasi selama 4 jam pada 65^0C. Dalam keadaan yang optimum, hasil pengekstrakan papain telah Berjaya diperolehi sebanyak 3.8018 μmole dengan 2.0910 Unit / ml pada sampel Selulase di rawat. Oleh kerana itu, potensi enzim papain dalam bidang perubatan dapat dipenuhi dengan menggunakan kaedah pendekatan enzim untuk pengekstrakan.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS/ABBREVIATIONS

A	-	Absorbance
Asn	-	asparagines
C	-	Celcius
cm	-	centimeter
Cys	-	Cysteine
F-C	-	Folin & Ciocalteu's Phenol Reagent
g	-	gram
g	-	gravitational acceleration
h	-	hour
His	-	histidine
M	-	Molarity
m	-	mili
m	-	meter
mg	-	milligram
min	-	minute
ml	-	mililiter
mm	-	millimeter
N	-	normality
nm	-	nanometers
rpm	-	revolutions per minute
Trp	-	Tryptophan
µ	-	micro
(v/v)	-	volume per volume
%	-	Percent
^{o}	-	degree

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Papaya Tree

Papaya (*Carica papaya* L.) belongs to the family of Caricaceae. It grows in Australia, Hawaii, Philippines, Sri Lanka, South Africa, India, Bangladesh, Malaysia and also other countries in tropical America. Papaya also known as tepayas by Kadazan Dusun community in East Malaysia, betik in Peninsular Malaysia, lechosa in Venezuela, pawpaw in Sri Lanka and papali in India (Rahman *et al*., 2008).

Papaya is a fast growing plant, rarely branching, semi-woody and short juvenile phase which is 3 to 8 months. When it starts flowering, it will continue to flower and produce fruit. Papaya is cultivated in tropical and subtropical regions. The papaya plants grow until 8–10 m in height with few branches and large leaves that bears yellow egg-shaped.Fruits 5–6 cm in diameter and 6–14 cm long with many seeds inside the fruit (Guillermo, Mario & Peter, 2009).

Many scientifics investigated the biological activities of various parts of *Carica papaya* L. such as fruits, shoots, leaves, rinds, seeds, roots or latex. There are many uses for the whole part of papaya especially in medical properties The papaya fruit contain some immune-stimulating and anti-oxidants agents while the unripe fruits and roots are used for abortifacient activity and also has shown bacteriostatic activity against the

human enteric pathogens. Besides that, the seeds used as a potential-testicular anti – fertility drug while the pulp is used for treating wounds and burns. For the latex and seeds, they are used in the care of gastrointestinal nematode infections and they also have shown anthelmintic activity .Then, for the leaves, they are used to relieve the symptoms of asthma and as vermifuge in treatment of gastric problems, fever and amoebic dysentery (Antonella *et al*., 2007).

1.1.1 Papaya leaves

Various parts of papaya include fruits, shoots, leaves, rinds, seeds, roots or latex have been traditionally used as ethnomedicine for a number of disorders, including cancer. There have been anecdotes of patients with advanced cancers achieving remission following consumption of tea extract made from papaya leaves **(**Morimoto *et al*., 2010).Papaya leaves constitute the most important part of the plant and play a major role in the anabolic activities by means of the so called "green pigment" or "chlorophyll", which they possess in abundance. Photosynthesis occurs within the chloroplast-containing mesophyll layer.

Papaya leaf juice is consumed for anti-cancer activity by people living with some anecdotes. Papaya leaf extracts have also been used for a long time as an aboriginal remedy for various disorders, including cancer and infectious diseases (Morimoto *et al*., 2010).The leaves are also used for relieving the symptoms of asthma and as a vermifuge, in the treatment of gastric problems, fever and amoebic dysentery. Methanolic leaf extract also demonstrated vasodilatatory and anti-oxidant effects, both implicated in the reduction of cardiovascular risks. Papaya leaves are also used in tropical alimentation cooked as a vegetable and in preparation of teas and infusions (Antonella *et al*., 2007).

The leaves of papaya contain many active components that can increase the total antioxidant power in blood. They also can reduce lipid peroxidation level, such as papain, chymopapain, cystatin, α-tocopherol, ascorbic acid, flavonoids, cyanogenic glucosides and glucosinolates(Morimoto *et al*., 2010).The content of papain, chymopapain, glycyl endopeptidase and caricain vary in fruit, leaves and roots (Rahman *et al*., 2008).

1.1.2 Papain

C. papaya plant is laticiferous because they contain specialized cells known as laticifers. Lactifiers secrete latex and are dispersed throughout most plant tissues. The papaya latex is well known for being a rich source of the four cysteine endopeptidases namely papain, chymopapain, glycyl endopeptidase and caricain. The content of papain, chymopapain, glycyl endopeptidase and caricain vary in fruit, leaves and roots. Commercially, papaya latex is harvested from fully-grown but unripe fruit. Ripe papaya contains less latex compared to green papaya possibly due to cessation of function or breakdown with age of the latexproducing cells (Rahman *et al*., 2008). When unripe, papaya contains the enzyme papain (EC 3.4.22.2), a cysteine protease. It is also cultivated for the proteolytic enzyme ‘papain’. Papain is a proteolytic enzyme from the latex in the leaf, the stem and the papaya's unripe fruits and possesses a stereospecific esterase activity on appropriate synthetic compounds (Wang, Chen &Wu, 1982).

The 3D structure of papain is well characterized. The enzyme consists of a single polypeptide chain made up of 212 amino acid residues and has a molecular weight of 23,400 Dalton. An interesting feature of papain molecular structure is that it is divided in the form of two distinct domains that are separated by a deep cleft which are L domain and R domain, forming a cleft with the active site (Prakash, Kumar & Sathish, 2009). L domain, which is mainly α-helical, is compromised of residues 10–111 and 208–212 while the R domain contains residues 1–9 and 112–207 and the key feature of the R

domain is its antiparallel β-sheet structure. In the cleft formed between these two domains are the active site residues, Cys-25, His-159 and Asn-178 (Prakash, Kumar & Sathish, 2007).

Latex from fruit is the most common part of the papaya plant being analyzed by scientists for its papain activity. Cysteine proteinases are used widely for protein digestion in the food and pharmaceutical industries. Latex of Carica papaya L., contains a mixture of cysteine endopeptidases such as papain (EC 3.4.22.2) ,chymopapains A and B (3.4.22.6) ,papaya endopeptidase III, papaya endopeptidase IV and endopeptidase U (caricain)(Salas *et al*., 2008).

1.1.3 Application of papain

There are many applications of the papain enzyme that extracted from papaya. The enzyme is used widely as meat tenderizer, and has also several other applications such as for defibrinating wounds, treatment of edemas and shrink proofing of wool (Rajni, Sarote, & Pawinee, 2006). Besides, the papain which is a sulfhydryl protease is one of the most commonly used enzymes in various industries including food, tanning and pharmaceutical industries (Prakash, Kumar & Sathish, 2009). The other uses of papain are cell isolation, breweries, food and pharmaceutical as digestive enzyme, leather, cosmetic and textile industries (Abraham & Sangeetha, 2006). A study show that the papain has been used in meat tenderizers and in face and hair care products. It is also increasingly being used in pharmaceutical preparations and in such diverse manufacturing applications as leather, wool, rayon and beer (Kamalkumar *et al*., 2007). Recently it is proven that the papain has multiple applications in the food industry such as a clarifier in beers, a meat tenderizer and in preparation of protein hydrolysates and the pharmaceutical industry like in treatments for osteoporosis, arthritis, vascular diseases and cancer (Santiago *et al*., 2009).

1.2 Problem Statement

The extraction of papain enzyme is commonly carried out by collecting the latex from green papaya by making incisions in the fruit surface .This damages the fruit and causes it to not meet the specification for commercial values. The decrease in commercial value using the unripe fruit can be reduced by another extracting method which is by using the leaves.

For extraction of papain enzyme, water or organic solvent such as methanol, ethanol and acetonitrile are commonly used as an extraction media. However, the use of aqueous organic solvent as extraction media can change the structural activity of papain due to the decreasing of hydrolytic activity and the number of active sites of papain. Therefore, in the extraction of papain enzyme from papaya leaves, water is applied as the extraction media. This is because water can maintain the structural stability of papain besides it is also very good extractive properties for polar substances compare to the organic solvent. The extraction of papain using water as extraction medium also avoid from toxicity due to the using of papain as the food application.

1.3 Objective

The objective of this research is to extract the papain enzyme from papaya leaves using enzyme assisted method and determine the papain activity.

1.4 Scope

In the extraction of papain enzyme from papaya leaves, it is focused on five scopes to obtain the optimum condition for the extraction.

The first scope of the research is to determine the optimum pH for extraction process which is divided into six parts: pH 3, pH 4, pH 5, pH 6, pH 7and pH 8.It is done to check whether the papain is acidic, neutral or basic enzyme.

The second scope is to determine the optimum concentration of enzyme (Cellulase) in extraction process. Five different concentrations are chosen which are 0.5 mg/ml, 1.0 mg/ml, 1.5 mg/ml, 2.0 mg/ml and 2.5 mg/ml.

The third scope is to determine the optimum solid to liquid ratio for extraction process and it is divided into five parts: 1:5g/ml, 1:10g/ml, 1:15g/ml, 1:20g/ml and 1:25g/ml.

The fourth scope is to determine the optimum incubation time for extraction process and it is divided into five parts: 1h, 2h, 3h, 4h and 5 h.

The last scope is to determine the optimum incubation temperature for extraction process. Five different temperatures are chosen which are $50^{o}C$, $55^{o}C$, $60^{o}C$, $65^{o}C$ and $70^{o}C$

1.5 Rationale and Significance

Extraction of papain is not only in the fruit but also in different plants tissue such as roots, stem, petiole and leaves. The extraction of papain enzyme from leaves is introduced in the research. Using this part of the papaya tree (leaves), it does not compete with another part of papaya tree especially fruit in producing end products.

In addition, leaves tissue yield the largest amount of papain enzyme compared to the other part of papaya tree which are stem, petiole and roots (Santiago *et al*., 2009). Then using the leaves byproducts also can manage disposal of tree byproducts for the benefits uses. These byproducts are generally disposed of in open areas. High transport costs limit any secondary uses and in most cases this waste is left to root, producing phytopathogens that cause ecological problems and pose a risk to human health (Santiago *et al*., 2009).

Besides that, using leaves can create waste to wealth application because of the papain enzyme contained in the leaf tissues can be used in industrial fields like pharmaceutical, brewery, meat, dairy, textile, photographic, optical, tanning, cosmetic, detergents, food and leather industry.

CHAPTER 2

LITERATURE REVIEW

2.1 Papaya Leaves

There are several constituents of papaya leaves including the fermenting agent myrosin, alkaloids, rutin, resin, tannins, carpaine, dehydrocarpaines, pseudocarpaine, flavonols, benzylglucosinolate, linalool, malic acid, methyl salicylate, another enzyme, chymopapain (latex and exudate), calcium, iron, magnesium, manganese, phosphorus, potassium, zinc, beta-carotene, B-vitamins and vitamins A, C and E. Papaya leave is an excellent treatment for digestive disorders and extremely useful for any disturbances of the gastrointestinal tract. Papain, the powerful enzyme in papaya, helps to dissolve and digest protein, thus easing stomach ailments and indigestion. Papaya leaves' enzyme, papain, not only digests protein, but it extends its activity to digesting carbohydrate.

The leaves of papaya contain active components that can increase the total antioxidant power especially in blood. It has been shown that the papaya leaves can reduce lipid peroxidation level such as papain, chymopapain, cystatin, α-tocopherol, ascorbic acid, flavonoids, cyanogenic glucosides and glucosinolates. Papaya leaf juice was consumed for anti-cancer activity with some anecdotes. Papaya leaf extracts are also used for a long time as an aboriginal remedy for various disorders, including cancer and infectious diseases (Morimoto *et al*., 2010).

Analyses show that the contents of papain, chymopapain, glycyl endopeptidase and caricain vary in fruit, leaves and roots (Rahman *et al*., 2008). Previously, the result shows that the leaf and fruit tissue had the highest protein contents of papaya harvest by-products (stems, unripe fruit, petioles and leaves). Leaf tissue also produced the highest total enzymatic extracts yield which probably corresponds to papain from Carica papaya L harvest by-product (Santiago *et al*., 2009).

2.2 Papain

Papain is a proteolytic enzyme preparation derived from fruits and other parts of papaya such as stem, petioles and leaves. It contained lactose or dextrin. The enzyme activity of papain is not less than 300,000 units per gram. Papain occurs as white to light yellow-brown powders. It is odorless or has slight characteristic odors. Papain is a proteolytic enzyme from plants and possesses a stereospecific esterase activity on appropriate synthetic compounds (Wang, Chen &Wu, 1982). Papain is applied as an enzyme in protein chemistry for the synthesis of many biologically active compounds. It is comprised of a single polypeptide chain which consists of 212 amino acid residues containing a total of 11 primary amino groups which in 10 Lys residues and 1 amino terminal. The enzyme folded into two domains, L domain and R domain forming a cleft with the active site. The first domain contains α-helix, while the second domain has a large content of β-sheet and a lesser amount of α-helix (Simon *et al*., 2009).

Papain is one of the sulfhydryl protease of carica papaya fruit. It is highly stable enzyme based on its interesting molecular structure and its many industrial applications. Its molecular structure consists of two distinct domains, with the active site in the groove between the domains. The first domain (residues 1–110) contains mainly α-helix, while the second domain (residues 111–212) has a large content of antiparallel β-sheet and a lesser amount of α-helix. There are five tryptophan (Trp) residues that are located in the two distinct domains. Three of the five tryptophan (Trp) residues are located in the first

domain (Trp7 in the β-sheet, Trp26 and Trp69 in the α-helical segments), and the other two tryptophan (Trp177 and Trp181) in the coil region of the second domain. One of the seven cysteine residues, which is Cys25, provides the free thiol group of the active site .The others six of cyteine residues which are Cys22–Cys63, Cys56–Cys95 and Cys153–Cys200 form disulfide bridges .Water molecules present in ice-like networks play an important role in the stability of the enzyme, especially at the domain–domain interface (Simon *et al*., 2006).

Papain was a highly active endolytic cysteine protease from Carica papaya .It is stable in harsh conditions and active at low and high temperatures. It also is less expensive than microbial enzymes besides has wide range of specificity and good thermal stability amongst other proteases. Because of such characteristics, the papain has high potential used in detergents. Papain molecules had a molecular weight of 23,000 Da and an isoelectric point of 9.5.Papain molecules consisted of a single peptide chain of 211 amino acid residues folded into two parts that form a cleft and having 11 lysine residues. Papain cleaves peptide bonds involving basic amino acids and it also has an esterase activity. It is used in breweries, food and pharmaceutical, leather, cosmetic and textile industries. The catalytic site of the enzyme contains a catalytic triad Asn-His+-Cys−, which exists as zwitterions. Papain can be chemically modified by different dicarboxylic anhydrides of citraconic, phthalic, maleic and succinic acids as Lysine residues are not a part of active site in papain. These anhydrides react with the ε-amino group of lysine residues and change its charges from positive to negative, leading to a shift in pH optima of the enzyme from 7 to 9(Abraham & Sangeetha, 2006).

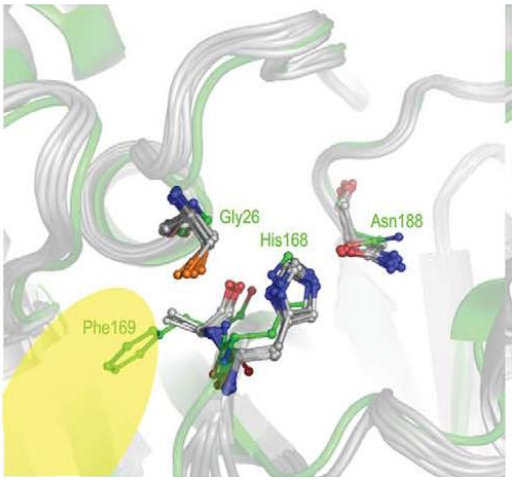

Figure 2.1: Active site residues, Cys-25, His-159 and Asn-178 of the papain between L and R domains (Gong *et al*., 2006)

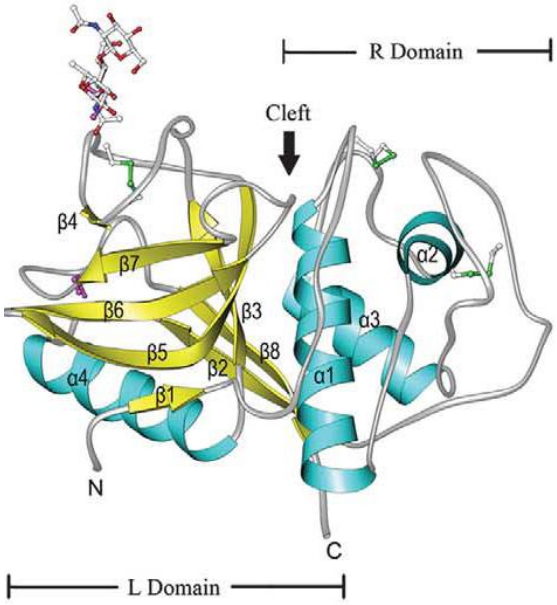

Figure 2.2: Active site in the cleft between these L and R domains (Gong *et al*., 2006)

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2.3 Hydrolysis pretreatment

The plant cells are surrounded by a complex cell wall matrix composed of carbohydrate molecules (cellulose, hemicelluloses, and pectic polysaccharides) as well as proteins. In order to achieve higher extraction, the activity for disrupting cell wall structure must be done to obtain the target product as intracellular product. Before the hydrolysis treatment, the samples must be treated into smaller particle such as grinding or crushing. During the hydrolysis treatment on the degraded surface, the middle lamellae is degraded causing cell tissue slowly and gradually lose cellular and sub-cellular organization as the walls and cytoplasm become disrupted (Silva *et al*., 2009).

There are many methods for hydrolysis treatment that have been published either in physical methods or chemical and physicochemical methods. The physical methods include disruption the cell in bead mill, using a rotor-stator mill, French press and ultrasonic vibration. For chemical and physicochemical method, it includes disruption the cell by using detergents, enzyme, solvents and osmotic shock. From the previous study, the enzyme-assisted extraction is a method applied to the study secondary metabolites releasing from biogenic materials. This kind of hydrolysis treatment has advantages of environmental friendship, high efficiency and easy operation process. It also has been represented as an alternative way for natural product extraction. Hydrolytic enzymes including cellulase, beta-glucosidase and pectinase, which are commonly used in extraction can interact on cell wall, break down its structural integrity so as to increase the releasing of intracellular products (Fu *et al*., 2009).

2.3.1. Enzymatic Assisted Extraction

Analysis shows that the cell wall degrading enzymes can improve the extraction of phenols from fruit skins. The enzyme assisted release of phenols from the cell wall matrix occurs via enzyme catalyzed hydrolytic degradation of the cell wall

polysaccharides that are presumed to retain the phenolics in the polysaccharide-lignin network by hydrogen or hydrophobic bonding. The use of cell wall degrading enzymes also increased the mass transfer of total phenols, with proteases having a particular increasing effect on the yield of chlorogenic acid. This study showed that phenols can be selectively extracted by varying the extraction conditions and by adding cell wall degradation enzymes. Another mechanism may be the direct enzyme catalyzed breakage of the ether and/or ester linkages between the phenols and the plant cell wall polymers. Fungal pectinases are the most widely used cell wall degrading enzymes in the fruit industry. In apple juice processing, the pectinases improve the press capacity and efficiency via viscosity lowering of the mash and are used for the juice clarification as well (Meyer, Pinelo & Zornoza, 2008).

In order to choose the best enzyme to increase the yields of taxanes notably, the activity of individual and complex enzymes was compared in this section. Enzyme-assisted extraction of paclitaxel and other taxanes, namely 7-xyl-10-DAT, 10-DAT, cephalomannine and 7-epi-10-DAT from needles of T. chinensis was carried out in present study. The effect of three hydrolytic enzymes which were Cellulase, Beta-glucosidase and Pectinase was compared. Cellulase catalyzes the breakdown of cellulose into glucose, cellobiose and higher glucose polymers. Pectinase has the ability to disintegrate pectic compounds and pectin while Beta-glucosidase breaks the beta-1,4 glucosidic linkages in glucosides. Although Beta-glucosidase was proved to be most effective for extracting taxanes from needles of T. chinensis, the cost is too high to afford in industry. Cellulose was chosen for the treatment of the needles by considering the economic effect (Fu *et al*., 2009)

2.3.2. Ultrasonic Assisted Extraction

Ultrasonic-assisted extraction is one of the important techniques for extracting the compounds from the vegetal materials and it is quite adaptable on a small or large

scale. The ultrasonic device is cheaper compared with other extraction techniques such as microwave-assisted extraction. Its operation is also much easier. The general ultrasonic devices are ultrasonic cleaning bath and ultrasonic probe system. When sonicating liquids at high intensities, the sound waves that propagate into the liquid media results in alternating high-pressure (compression) and low-pressure (rarefaction) cycles, with rates depending on the frequency. During the low-pressure cycle, high-intensity ultrasonic waves create small vacuum bubbles or voids in the liquid. When the bubbles attain a volume at which they can no longer absorb energy, they collapse violently during a high-pressure cycle. This phenomenon is termed cavitations. When these bubbles reach resonance size, they collapse releasing mechanical energy in the form shock waves. The shock waves disrupts cell in the suspension.

From the previous research, an ultrasonic probe system was chosen as the ultrasonic device to extract the intracellular product. In this study, the feasibility of the extraction of epimedin C from fresh leaves of Epimedium using ultrasonic probe system was demonstrated. The high extraction yield of epimedin C was obtained under an optimum extraction condition. The high yield was obtained form the extraction temperature of 50 °C, methanol concentration 60% (v/v), ratio of liquor to solid 30 ml $g^{-}1$, and ultrasonication time for 15 min. Ultrasound could result in the disruptions of leaf tissues and cell walls, which enhanced the mass transfer of the solvents into the leaf materials and the soluble constituents into the solvents (Wang *et al*., 2009)

Previously, the study introduced ultrasonication in the traditional enzymatic release of protein- and phosphate bound thiamin and riboflavin in the determination of vitamin B1 and B2 in foods. Unfortunately, the ultrasonication process did not show any effect on the efficacy of the enzymes but enabled the enzymatic treatment to be performed within 1 h, as a replacement for 4–18 h incubation for vitamin B1 and 18 h incubation for vitamin B2 in the standardized methods (Jakobsen, 2008).

2.4 Method of Extraction

Many extraction methods including microwave-assisted extraction, Soxhlet extraction, Percolation, bubble column extraction (BCE), Supercritical fluid extraction (SFE), reverse micellar extraction and Heat-refluxing extraction had been reported.

2.4.1 Heat-refluxing extraction

A conventional method of heating-refluxing extraction using ethanol–water (80:20, v/v) was performed .According to the preliminary investigation, target compositions were extracted by adding 20 g of pigeonpea leaves into 400 ml of solvent in a round bottom flask. The extraction was employed to optimum condition of 65 ^{0}C for 2 h under magnetic stirring at 500–700 rpm. The extracting solution was filtered by membrane filtration and analyzed by HPLC (Fu *et al*., 2009)

2.4.2 Soxhlet extraction, Percolation, bubble column extraction (BCE)

In the extraction of solanesol from tobacco, the extraction methods using Soxhlet extraction, Percolation and bubble column was compared. A bubble column reactor is basically a cylindrical vessel with a gas distributor at the bottom. The gas is spurge in the form of bubbles into either a liquid phase or a liquid–solid suspension. In this device, bubbles were introduced into liquid–solid system (made of the extraction solvent and material) to increase the turbulence in the medium and transfer coefficient (Zu, Zhao & Li, 2009).

For this experiment, percolation was conducted using a glass column. Material was packed into the column and the solvent was added continuously to percolate through the material packing and collected. The flow rate was set at and lastly the infiltrated

solution was filtered through a 0.45 µm mmembrane filter before chromatographic analysis (Zu, Zhao & Li, 2009).

A conventional Soxhlet extraction was used. The materials were ground and packed into a filter paper. This cartridge was placed inside the Soxhlet extractor, which was placed on top of a round-bottomed flask filled with the solvent. The system was boiled using a bath boiler until the extraction was completed. After filtration, the extract was fixed volume in 10 ml volumetric flasks prior to chromatographic analysis (Zu, Zhao & Li, 2009).

Result shows that the yields of solanesol by the above three extraction methods (percolation, Soxhlet and BCE) are similar, but the extraction time varies with the different extraction methods. Percolation, Soxhlet extraction and BCE need 24 h, 6 h and 54min respectively to complete the extraction. BCE reduces the extraction time, proving it is a fast and efficient method for the extraction of solanesol (Zu, Zhao & Li, 2009).

2.4.3 Supercritical fluid extraction (SFE)

From the previous study of extraction of rose geranium oil, the supercritical fluid extraction (SFE) using supercritical CO_2 was used to produce a natural extract from Portuguese-grown geranium as a high quality material for application in perfumery. As SFE uses gentle operating conditions (low temperature, close to ambient), the SFE extract has been recognized as having a superior quality in terms of odor and taste, with fresher characteristics and resembling more to its natural source, especially when compared with distilled oils. Unfortunately, SFE is a very expensive technology due to the high investment costs and safety precautions of working at high pressures. SFE is profitable only when applied to high-added value, to obtain ultra-pure products or if impose by regulatory restrictions on residues .Pressure and temperature are the most

important operating parameters that affect the supercritical fluid selectivity, yield and extraction rate, especially in the vicinity of the critical point. Other operational parameters are the pre-treatment of plant material, solvent flow rate, extraction time and design of extractor (relation height/width). The physical properties of the solute, namely vapour pressure, polarity and molecular weight, affect its solubility in the supercritical fluid (Mata, Gomes & Rodrigues, 2007).

2.4.4 Reverse micellar extraction

Extensive studies have been done on reverse micellar extraction of proteins using an anionic surfactant, bis (2- ethylhexyl)sulfosuccinate (AOT) .The distribution of proteins between the micellar phase and an aqueous phase is largely determined by the environments of bulk aqueous phase, i.e., pH, ionic strength and type of salt. Parameters related to the organic phase also affect the partition of protein, such as the concentration and type of surfactant, presence of co-surfactant and type of solvent. By controlling these parameters, the extracted fraction can be varied via variations of protein–micelle electrostatic, hydrophobic and steric interactions.

In order to employ reverse micelles for protein separations, the micelles should exhibit two characteristic features. Firstly, they should be capable of solubilizing proteins selectively. This protein uptake into the reverse micelles is referred to as forward extraction process. Second, it should be possible to release the protein from the reverse micelles so that a quantitative recovery of purified protein can be achieved. This is referred to as the back extraction process.From the result about 60–65% of papain was forward extracted (1st step) without much difficult (Juang & Mathew, 2005).

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